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Comparative Quality Of Mice Heart Histology Using NBF 10% and Honey 13% with Time Variation As Fixation Agent

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ABSTRACT The fixation stage is carried out to maintain the cell structure of the tissue to resemble living tissue and prevent tissue damage, thus facilitating the process of macroscopic and microscopic analysis. The fixative solution used for standard preservation in anatomical pathology laboratory examinations is Neutral Buffered Formalin (NBF) 10% which has the advantage of a pH that is close to neutral and effective in fixating soft tissue for 12-24 hours. However, 10% NBF fixation solution is carcinogenic and has a high toxicity impact on laboratorian health. There is an alternative to using a natural fixative solution in the form of honey, which contains fructose and glucose and has an acidic pH that has antimicrobial, antioxidant and antiautolysis properties. In addition, honey has also been widely used as a preservative because it is easy to obtain, affordable, and without toxicity effects. The purpose of this study was to determine the comparison of the quality of histology preparations of mice heart tissue fixed with 10% NBF and 13% honey with time variations. The method used was a laboratory experiment conducted in November 2024-April 2025. The sample used was the heart of healthy male mice. The number of sample preparations used was 27 sample preparations. Data analysis through scoring and statistically processed using non-parametric Kruskal-Walis test, if there is a difference followed by Mann-Whitney test. The results showed that fixation with 13% honey solution with a time variation of 24 hours has the same comparison of preparation quality results with 10% NBF solution for 24 hours, based on the Mann-Whitney test results of 0.634 ($p > 0.05$) for 13% honey solution for 24 hours. These findings suggest fixation with 13% honey solution for 24 hours is effective as an alternative fixation solution with natural ingredients that are safe in fixating tissue.

INDEX TERMS Fixation, Neutral Buffer Formalin 10%, Honey, Mice, and Heart.

I. INTRODUCTION

Histopathologic examination is the gold standard in determining the diagnosis by looking at the changes that occur in the cell morphology of a tissue that has been prepared [1][2]. Histopathology preparations go through several processes, namely fixation, tissue maturation, embedding, cutting tissue with a microtome, and staining with hematoxylin eosin [3]. The initial stage of fixation is an important process for good and clear microscopic analysis results, because it plays a role in maintaining the cell structure of tissues to be like living tissues, preventing autolysis and tissue damage [4]. The fixative solution used as a standard of preservation in histological examination is Neutral Buffered Formalin (NBF) 10% [5].

Neutral Buffered Formalin (NBF) 10% is a standard fixation solution that has the advantages of being able to maintain tissue components, harden the tissue so as to facilitate the cutting process, have a pH that is close to neutral, and can be stored for a long time [6]. The standard time required for the fixation process is 24 hours, this is a shortcoming of fixation using NBF 10% [7]. According to the Occupational Safety and Health Administration (OSHA) and Environment Health and Safety Information (EHSI) formalin has toxic properties and as a class 1 carcinogenic material that has the potential to cause severe eye damage, respiratory irritation, drowsiness, dizziness, allergic skin reactions, and cancer [8][9]. Despite NBF 10% eficacy, its toxicity raises concerns that encourage the exploration of fixation materials with substances that are safe for the environment, and safety for humans, with the natural ingredients being pure honey [10].

Honey as an alternative to formalin in the fixation process with various properties from its content and has been used as a natural sweetener for more than six thousand years [11][12]. Honey has a low pH concentration, acidic honey can function as an antimicroorganism and antiautolysis, so honey has fixative properties as a substitute for NBF 10% [13][14]. The very high fructose and glucose content in honey causes the solution to be very hypertonic, this property will cause the lysis of bacteria due to severe dehydration due to the osmosis effect [15]. Thus, the use of natural honey as an alternative to formaldehyde can help reduce exposure to NBF 10% for the health of medical personnel[16][17].

Based on the research that has been done, pure honey with concentrations of 10%, 15%, 20%, 50%, 70%, 90%, and 100% is able to become a fixation material. Honey 10% and 15% showed the best results. This study aims to test the presence of honey concentration between the range of 10%-

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15% in order to get better quality preparation results [18][19]. In addition, to refine previous research from the use of 10% concentration honey that has not obtained perfect results and it is expected that there is a concentration of less than 15% that provides fixation results with better preparation analysis in an optimal time, namely the use of 13% concentration honey with time variations for 12 hours and 24 hours. This research is expected to be a solution to reduce the negative impact of using formalin, as well as provide a safer, cheaper, and environmentally friendly method of making histology preparations. This study used a laboratory experimental design with quality assessment of preparation results to compare control and treatment preparations. The contributions of this research include:

1. Development of knowledge about alternative natural fixation materials that can maintain tissue structure and quality.
2. Providing comparative information on the effect of fixation time on tissue structure.
3. Providing recommendations for fixation materials and time in improving the quality of histology preparations, which are safe and more cost efficient.

II. METHOD

This study is included in the type of laboratory experimental research to determine the comparison of fixation solutions using NBF 10% solution for 24 hours and 13% honey solution with variations of 12 hours and 24 hours on the quality of histology preparations of mice heart tissue. The selection of this methodology was carried out to directly test the use of natural and chemical materials by comparing the results of the quality of the preparations [20].

A. STUDY SETTING

This research was conducted at the Faculty of Veterinary Medicine, Universitas Airlangga, Cytohistotechnology Laboratory, Department of Medical Laboratory Technology, Poltekkes Kemenkes Surabaya, and Farma Veterinary Center (PUSVETMA). This research was conducted from November 2024 to May 2025.

B. PARTICIPANTS AND SAMPLING METHOD

The variables used in this study are 2 variables, independent variables and control variables.

TABLE 1
Determination Of Variables And Treatment Groups

Control Variables	Independent Variables
Quality of histological preparations of mice heart tissue (*Mus musculus*)	Honey 13% solution as 12 hours and 24 hours time variation
Treatment Groups	
1. Honey 13% solution for 12 hours	
2. Honey 13% solution for 24 hours	
3. NBF 10% solution for 24 hours (as a gold standard)	

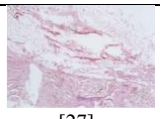
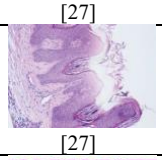
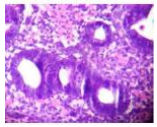
The sample used for this study is the heart organ of laboratory mice (*Mus musculus*) with the criteria of healthy, no treatment, weighing 20-40 grams, male sex, active movement, routine feeding for 7 days, and cage care for every 3 days [21]. The use of mice heart samples is to see the ability of honey fixative solution in maintaining structural and muscle components in the mice heart [22][23]. Determination of the total samples using the Federer formula because the formula is designed for experimental research using treatment groups and results in 9 samples in each treatment. Total of 9 samples has fulfilled the minimum sample required so that the effect being studied can be detected statistically with a valid level of precision and validity, and is able to reveal real differences between treatments on the quality of preparations. So the total sample of the 3 treatments is 27 preparation samples [24].

The flow in this study is the preparation of a 13% honey solution derived from the honeycomb squeezing process of mellifera bees. The weight of the mice heart organ ± 1 gram requires 20 mL of fixation solution, because the ratio of fixation volume is 1: 20 [2].Healthy male mice that have been treated for 7 days and dissected to take heart tissue at the Faculty of Veterinary Medicine, Airlangga University, after which fixation is carried out with 10% NBF solution for 24 hours and in 13% honey solution for 12 hours and 24 hours at room temperature (24-30ºC). Mice heart tissue samples that have been fixed with both solutions are crossed and continued with tissue processing (dehydration, clearing, infiltration) [25]. Then the mice heart tissue samples were embedding, sectioning, staining with hematoxylin eosin staining, and mounting carried out at the Cytohistotechnology Laboratory, Department of Medical Laboratory Technology, Poltekkes Kemenkes Surabaya. Histological preparations of mice heart tissue were observed with a 100x objective lens magnification microscope and validated by one veterinarian at the Farma Veterinary Center (PUSVETMA). Then the results of the quality of histological preparations were analyzed by statistical processing and the conclusion was obtained from the comparison.

C. DATA COLLECTION PROCEDURE

This study uses categorical assessment with based on predetermined criteria to determine the comparison of the quality of the results of tissue preparations fixed using 10% NBF solution and 13% concentration honey solution with time variations for 12 hours and 24 hours. The following is a table of preparation assessment criteria [26] :

TABLE 2
Criteria for Assessment of Preparation Quality.

Scoring	Assessment Criteria			Example Image
	Cell Nucleus	Cyto plasm	Uniformity of Color	
1 Not Good	Blue color is not clear	Red color is not clear	Overall not evenly colored	 [27]
2 Good Enough	The blue color is quite intense	Red color is quite even	Overall quite clear and quite readable	 [27]
3 Good	Clear blue color	Clear red color	Overall evenly colored	 [27]

D. DATA ANALYSIS

The data obtained is the result of a microscopic description of the quality of mice heart tissue preparations that have been

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categorized based on the assessment of predetermined criteria. The data were analyzed statistically with the Kruskal-Wallis test because this study has used categories in the form of a nominal scale with the criteria 1 is not good enough, 2 is good enough, 3 is good. This experimental research also uses sample data (<30) and categorical data, so it is included in non-parametric. Data processing uses non-parametric statistics with the number of treatment groups more than 2, namely 3 treatments using the Kruskal-Wallis test analysis to compare any differences in data test results [28].

Data analyzed by the Kruskal-Wallis test, if it shows the Asymp.Sig result ($p < 0.05$) then there is a difference between the three treatment groups so that it can be continued for the Mann-whitney test. Mann-whitney test is done to see which group there is a significant difference by testing each control group and 1 treatment group, and if the results of the 2 treatment groups test ($p > 0.05$) then the treatment group shows no difference with the control group.

III. RESULTS

A. *HISTOLOGICAL QUALITY ASSESSMENT RESULTS*

Based on the results of research that has been conducted in April 2025 regarding the comparison of the quality of the results of mice heart tissue preparations fixed with NBF 10% and honey 13% with time variations. The material used for the substitute fixative solution is a honey solution derived from mellifera honeycombs and the samples used as research material are mice heart organs (Mus musculus) with predetermined criteria that can be used as research material. The criteria for assessing the quality of preparations in this study are seen based on the quality of coloring of the nucleus that looks blue, the cytoplasm that looks red, and the uniformity of coloring in the entire mice heart organ preparation. The following is the data on the results of the validation of the preparation assessment that has been carried out:

TABLE 3
Results of Comparative Assessment of the Quality of Histological Preparations of Mouse Heart Tissue Fixed with 10% NBF and 13% Honey with Time Variations.

Slide/Sample Replication	Heart Histology Quality Assessment of Mice (*Mus musculus*) with Score		
	NBF 10% Control 24 Hours	Honey 13% 12 Hours	Honey 13% 24 Hours
1	3 (Good)	2 (Good Enough)	3 (Good)
2	3 (Good)	3 (Good)	3 (Good)
3	3 (Good)	2 (Good Enough)	3 (Good)
4	3 (Good)	2 (Good Enough)	3 (Good)
5	3 (Good)	2 (Good Enough)	3 (Good)
6	3 (Good)	2 (Good Enough)	3 (Good)
7	3 (Good)	2 (Good Enough)	2 (Good Enough)
8	3 (Good)	3 (Good)	2 (Good Enough)
9	1 (Not Good)	2 (Good Enough)	3 (Good)

Score Description:
1: the quality of the preparation is not good as seen from the cell nucleus, cytoplasm, color uniformity
2: the quality of the preparation is quite good as seen from the cell nucleus, cytoplasm, color uniformity
3: the quality of the preparation is good as seen from the cell nucleus, cytoplasm, color uniformity

Based on the TABLE 3, these findings are illustrated in the form of a bar chart to facilitate visual comparison across treatment groups.

TABLE 4
Percentage of Histology Preparation Quality Assessment Results

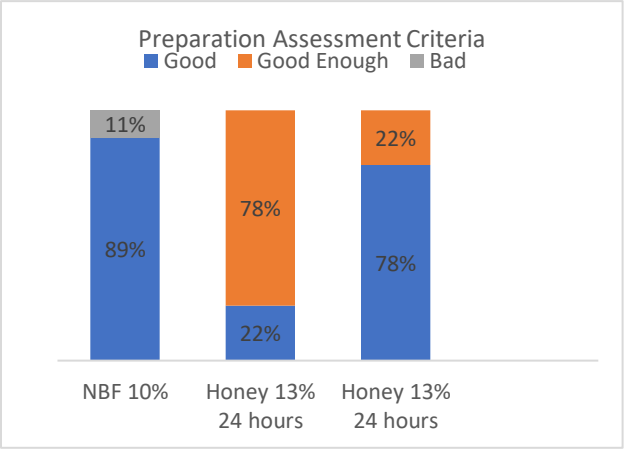
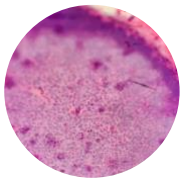
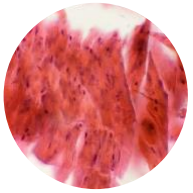
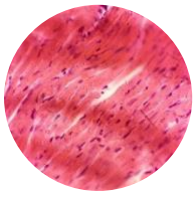


TABLE 5
Microscopic View of Research Results using honey 13% as fixation material.

Scoring	Description	Image Result
1 (Not Good)	- Cell nucleus not clearly visible blue color - Cytoplasm is not clearly visible red color - Color uniformity in the preparation is uneven and not clearly visible	
2 (Good Enough)	- The nucleus of the cell is quite clearly colored blue - Cytoplasm looks quite clear red color - Color uniformity looks evenly quite clear	
3 (Good)	- The cell nucleus is clearly colored blue - Cytoplasm is clearly visible red color - Color uniformity is clearly visible evenly	

Based on TABLE 4, it can be seen the description of mice heart tissue preparations that have been observed under a microscope based on the preparation quality assessment criteria in TABLE 2 The results of the assessment are seen from the cell nucleus, cytoplasm, and color uniformity obtained there were 16 preparations that had a total score of 3 (good), 9 preparations that had a total score of 2 (fair), and 1 preparation that had a score of 1 (not good) from all treatments. The following is a description of the results of the

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assessment of the quality of mice heart tissue preparations based on the coloring of samples viewed under a microscope:

B. *DATA ANALYSIS*

The data in this experimental study used categories in the form of a nominal scale with criteria assessment, samples (<30), and 3 treatment groups, so the *Kruskal-Wallis* test analysis was used. The following are the results of the *Kruskal-Wallis* statistical test:

TABLE 6
***Kruskal-walis* Statistical Test Results.**

Fixation Method	*Kruskal-walis*		Description
	N	*Asymp. Sig*	
NBF 10% Solution	9	0,019	There is a difference
Honey 13% Solution 12 hours	9		
Honey 13% Solution 24 hours	9		

Based on **TABLE 6** above, the results obtained Asymp. Sig 0.019 which shows the value of *Asymp. Sig* <0.05 which means that there is a significant difference in the histology of mice heart tissue preparations between treatment groups. Further analysis is needed to determine the specific location of differences between treatment groups, which is continued with the *Mann-Withney* test. the following are the results of the *Mann-Withney* statistical test:

TABLE 7
***Mann-Withney* Statistical Test Results.**

Treatment Group	*Mann-Withney*		Description
	N	*Asymp. Sig* (2 tailed)	
NBF 10% Solution and Honey 13% Solution 12 hours	18	0,018	There is a difference
NBF 10% Solution and Honey 13% Solution 24 hours	18	0,634	There is no difference

Based on the results of the Mann-Withney test above, it can be seen that fixation with 13% honey solution with a time of 24 hours has the same quality of preparation with 10% NBF control solution for 24 hours as evidenced by the results of *Asymp. Sig* > 0.05 which means there is no difference.

IV. DISCUSSION

A. *INTERPRETATION OF THE RESULT*

This experimental study discusses the comparison of the quality of histology preparations of mice heart tissue fixed with NBF 10% for 24 hours and honey 13% with time variations for 12 hours and 24 hours with hematoxylin eosin staining. The aim is to get a better quality histology preparation with a new concentration and in the best time optimization. This preparation uses heart tissue samples of 10 male mice weighing 20-35 grams, and aged 6-8 weeks.

The The fixation stage carried out properly according to the standard will produce good quality and the cell nucleus, cytoplasm are clearly visible, and provide even color contrast [29]. Fixation is carried out with a standard preservation solution in histological examination is Neutral Buffered Formalin (NBF) 10% which has the advantage of having a pH that is close to neutral, and can be stored for a long time [30]. However, it also has the potential to cause severe eye damage, skin disorders, respiratory irritation, drowsiness, dizziness, allergic skin reactions, and cancer. Carcinogenic ingredients from formalin can be replaced with alternative natural ingredients, namely honey, which has been done in previous studies that honey is able to fix tissues because it contains antimicrobial compounds and high osmotic properties derived from 70-80% sugar content so that it can inhibit autolysis and tissue degradation [13]. Natural honey has a low pH, which is acidic (3-4), in this study the use of 13% honey with distilled water solvent which makes the pH close to neutral, namely 6 can reduce the risk of tissue damage due to acidity in mice heart tissue [17].

Based on the results of research that has been obtained, the assessment of comparing histology preparations of mice heart tissue that have been fixed with NBF 10% solution for 24 hours and honey 13% solution with time variations for 12 hours and 24 hours shows that honey 13% solution is proven to fix the tissue well. Fixation with NBF 10% for 24 hours produces preparations with optimal hematoxylin eosin staining, characterized by a blue nucleus (hematoxylin) and homogeneous pink cytoplasm (eosin). Preparations fixed with honey 13% solution with different fixation time treatments and then followed by hematoxylin eosin staining also produced different staining qualities of cell nuclei, cytoplasm, and color uniformity [31].

The results of the assessment of the quality of mice heart preparations that have been validated and scored for each preparation will be followed by data analysis using the Kruskal-Wallis statistical test. The results of the Kruskal-Wallis statistical test on the three treatments showed that therewere significant differences in all treatments. Then proceed with a more specific test to see the difference in treatment between groups, namely the Mann-Withney statistical test. The results of Mann-Withney statistical test data by testing each control group and 1 treatment group showed that the 13% honey solution for 12 hours with 10% NBF had a significant difference. For 10% NBF solution and 13% honey solution for 24 hours, the results showed no significant difference.

B. *COMPARISON OF PREPARATION RESULTS*

The treatment of two variations of fixation time using honey 13% solution for 24 hours showed better results than fixation with honey 13% solution for 12 hours. This is obtained from the results of the assessment of preparation quality criteria that have been determined based on **TABLE 1**. Preparations fixed with honey 13% solution for 24 hours get good quality score results and results that are almost the same as the quality of preparations fixed with NBF 10% for 24 hours. Preparations fixed with honey 13% solution for 12 hours still get pretty good preparation quality results and it does not mean failure in fixing the tissue.

Based on the results of microscopic observations and quality assessment of mice heart tissue preparations with fixation using honey 13% solution for 24 hours resulted in fixation equivalent to NBF 10% in terms of clarity of the nucleus (cell nucleus) which is blue and cytoplasmic integrity which is clearly visible pink and almost good color uniformity in the whole preparation. The hygroscopic nature of honey (high fructose content) allows stable penetration in fixing the

tissue without causing excessive shrinkage [15]. The content of honey in the form of ascorbic acid and various vitamins, carbohydrates, and minerals makes honey have a low pH so that low pH fixatives are suitable for staining cell nuclei [17].

The results of microscopic observations and quality assessment of mice heart tissue preparations with fixation using honey 13% solution for 12 hours showed quite good results. Staining of the cell nucleus has been seen quite well blue in some preparations, but for cytoplasmic staining and color uniformity is still not good, only 2 preparations are seen that have good quality. In the treatment of time variation for 12 hours, it has not produced preparations equivalent to NBF 10% because honey is also a natural material that requires a longer time for the solution to bind to the tissue. The quality of the preparation also depends on the time during fixation. The standard fixation time for 10% NBF solution ensures perfect penetration and fixation reaction in the range of 12-24 hours. In accordance with the performance of fixation time, the first 6 hours of the solution will diffuse into the tissue to form methylene hydrate, then the methylene hydrate formed binds to the protein chain of the cell to form a reactive hydroxymethyl chain for 24 hours. At honey 13% fixation time for 12 hours has not produced a good preparation because honey is still in the process of diffusing into the tissue and forming cross-links with proteins so that there is a slight inhomogeneity of cytoplasmic staining due to uneven penetration [32].

This study confirmed that a 13% honey solution can fix histological tissues so that cell nuclei and cytoplasm are preserved comparable to 10% NBF, without the associated risk of toxicity. In accordance with the aims and objectives made, this experimental study succeeded in providing an update in the presentation of the use of honey between 10%-15% to find a better quality than previous studies that have been carried out, namely fixation with 10% and 15% honey solutions . When honey is dissolved with a low concentration, the pH will increase, and enzyme activity will also increase with the release of its antiseptic properties but does not damage the tissue [24], so it can be proven that a lower presentation than 15%, namely 13% honey, has a good quality of preparation results in maintaining cell morphology comparable to 10% NBF.

C. IMPLICATIONS

The following are the biological implications and practical applications of this research :

1. These findings indicate that the 13% honey solution has the ability to maintain cell integrity during the 24-hour fixation period suggesting that its bioactive components (such as glucose, enzymes, and natural antibacterial properties) play a role in the stabilization of biological structures. Honey exhibits the ability to protect the nucleus and cell membrane, which is important for histopathological diagnostic interpretation and results The preparations from this study exhibited clean morphology, supporting their potential application in tissue studies that require high precision.
2. Honey as an alternative fixative is promising for anatomical pathology laboratories with limited access to formalin or in educational environments that require safe and environmentally friendly materials.
3. Fixation using honey solution can be applied for safer tissue sampling in animals, especially in the context of small or field veterinary clinics.
4. The use of honey solution as a fixation material is more environmentally friendly and low in toxicity. In contrast to carcinogenic formaldehyde, honey offers a safer alternative for users and the environment, as well as cost and availability that is easily obtained locally and does not require special handling in storage.

D. LIMITATION OF HONEY

1. Honey as a natural material also requires almost the same time as NBF to fix the tissue
2. Honey as a fixation material cannot be preserved for a long time because honey as a natural material cannot harden the tissue so that the tissue can be destroyed if stored for too long.

V. CONCLUSION

This study aims to determine the comparison of the quality of histology preparations of mice heart tissue fixed with NBF 10% and honey 13% with time variations for 12 hours and 24 hours. From these objectives it is expected to get a better quality of histology preparation with a new concentration and in the best time optimization. The results showed that the alternative use of this natural fixation material had a significant positive impact. The results of the quality of histological preparations of mice heart tissue fixed with 10% NBF solution for 24 hours as a control resulted in 8 preparations showing optimal Hematoxylin Eosin staining results, characterized by clear blue cell nuclei, clear pink cytoplasm, and evenly distributed color uniformity.

In the results of the quality of histological preparations of mice heart tissue fixed with 13% honey solution with a time variation of 12 hours showed the results of the majority of fairly good preparates as many as 7 preparations with blue cell nuclei, pink cytoplasm and uneven color uniformity, While the variation of fixation time for 24 hours shows the results of the majority of 7 good preparations with clear blue cell nuclei, clearly visible pink cytoplasm, and evenly distributed color uniformity. The quality of preparations fixed with 13% honey solution for 24 hours gets better preparation results compared to the variation of fixation time for 12 hours. This study proves that 13% honey solution for 24 hours protocol is effective as a fixation solution with natural materials that can serve as an alternative with the latest concentrations that are non-toxic, cost-effective in histopathology laboratories, easily available, environmentally friendly, and guarantee wider tissue testing. Future research is expected to involve different tissue samples with larger and more diverse sizes such as samples with indications of abnormalities in order to increase the generalizability of the findings, because each sample has different characteristics. It could also be accompanied by a longer fixation time in order to assess whether the increase in knowledge is sustainable over time and produce alternatives to the use of other natural materials as fixation materials with optimal concentrations and time.

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DATA AVAILABILITY

No datasets were generated or analyzed during the current study.

AUTHOR CONTRIBUTION

Amirah Putri Zerlina conceptualized and designed the research, conducted the research procedures, performed data collection, participated in data analysis and interpretation. Juliana Christyaningsih contributed from the beginning in drafting the title to the background, provided advice on sampling and research procedures, assisted in analyzing data and interpreting research results, and contributed to the writing and revision of the manuscript. Ratno Tri Utomo contributed to the development of insights, provided advice on the selection of research materials, supervised the implementation of the research, assisted in data interpretation and provided many suggestions on good and correct manuscript writing and revision. Suliati contributed and assisted in interpreting the data and provided critical feedback on the manuscript, participated in providing guidance and suggestions in the preparation of data results and editing of the manuscript. All authors reviewed and approved the final version of the manuscript, and agreed to take responsibility for all aspects of the work to ensure integrity and accuracy.

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